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(54) Title: DETECTION METHOD FOR C-RAF-1 GENES

(57) Abstract

The present invention relates to: (1) a method of identifying an individual at an increased risk for developing cancer, (2) a method for determining a prognosis in patients afflicted with cancer, and (3) a method for determining the proper course of treatment for a patient afflicted with cancer; comprising: amplifying a region of the c-raf-1 gene.

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DETECTION METHOD FOR C-RAF-1 GENES

Field of the Invention

The present invention relates to (1) a method of identifying an individual at an increased risk for developing cancer, (2) a method for determining a prognosis of patients afflicted with cancer, and (3) a method for determining the proper course of treatment for a patient afflicted with cancer.

Background Information

Lung cancer claims more lives in the United States than any other neoplasm (R.S. Finley, Am. Pharm. NS29, 39 (1989)), and of the various forms lung adenocarcinomas have one of the worst prognoses (T.P. Miller, Semin. Oncol. 17, 11 (1990)). The incidence of adenocarcinoma of the lung (ACL) in the United States is also quickly rising (I. Linnoila, Hematol. Oncol. North. Am. 4, 1027 (1990); J.B. Sorensen, H.H. Hansen, Cancer Surviv. 8, 671 (1989)). In order to gain insight into this complex and deadly disease, a model system for its study has been developed. For such a model to provide clinically relevant data several criteria must be met. The tumors produced should be histologically equivalent to their human counterparts, tumor induction must be reliable and reproducible, and the numbers generated must be great enough to provide statistical significance. To satisfy these conditions a system has been created which uses two inbred mouse strains (NFS/n and AKR) along with

transplacental exposure to the potent carcinogen N-ethyl-N-nitrosourea (ENU) and promotion with the antioxidant butylated hydroxytoluene (BHT). The resulting tumors were examined for altered expression or structural mutations of genes implicated in lung tumor development such as *ras*, *myc*, and *raf* oncogenes (C.D. Little et al., *Nature* 306, 194 (1983); P.E. Kiefer et al., *Cancer Res.*, 47, 6236 (1987); E. Santos et al., *Science* 223, 661 (1984); S. Rodenhuis, *N. Engl. J. Med.* 317, 929 (1987); M. Barbacid, *Eur. J. Clin. Invest.* 20, 225 (1990); U.R. Rapp et al., *J. Int. Assoc. for the Study of Lung Cancer* 4, 162 (1988); M.J. Birrer et al., *Ann. Rev. Med.* 40, 305 (1989); G. Sithanandam et al., *Oncogene* 4, 451 (1989)).

raf proto-oncogenes are evolutionarily highly conserved genes encoding cytoplasmic serine/threonine specific kinases, which function in mitogen signal transduction (reviewed in U.R. Rapp et al., *The Oncogene Handbook*, T. Curran et al., Eds. (Elsevier Science Publishers, The Netherlands, 1988), pp. 115-154; U.R. Rapp, *Oncogene* 6, 495 (1991)). The three known active members in the *raf* family encode phosphoproteins of similar size (72/74 kD for Raf-1; 68 kD for A-Raf-1, and 74 kD for B-Raf (U.R. Rapp et al., in *Retroviruses and Human Pathology*, R. Gallo et al., Eds. (Humana Press, Clifton, New Jersey 1985), pp. 449-472; T.W. Beck et al., *Nucleic Acids Res.* 15, 595 (1987); G. Sithanandam et al., *Oncogene* 5, 1775 (1990)). Raf-1 was first identified as the cellular homologue of v-*raf* (H.W. Jansen et al., *Nature* 307, 218 (1984)), the transforming gene of 3611 MSV (U.R. Rapp et al., *J. Virol.* 45, 914 (1983); U.R. Rapp et al., *Proc. Natl. Acad. Sci. USA* 80, 4218 (1983)). Amino acid comparisons of *raf* family genes shows three conserved regions [CR1, CR2, CR3] (T.W. Beck et al., *Nucleic Acids Res.* 15, 595 (1987)); CR1 is a regulatory region surrounding a Cys finger consensus sequence, CR2 is a serine/threonine rich region, and CR3 represents the

kinase domain. Raf-1 has been mapped to chromosome 3p25 in humans (S.J. O'Brien et al., Science 223, 71 (1984)), and this region has been found to be frequently altered in small cell lung carcinoma (SCLC) (J. Whang-Peng et al., Cancer Genet. Cytogenet. 6, 119 (1982); J.M. Ibson et al., J. Cell. Biochem. 33, 267 (1987)), familial renal cell carcinoma (A.J. Cohen et al., N. Engl. J. Med. 301, 592 (1979); G. Kovacs et al., Int. J. Cancer 40, 171 (1987)), mixed parotid gland tumors (J. Mark et al., Hereditas 96, 141 (1982)), and ovarian cancer (K. Tanaka et al., Cancer Genet. Cytogenet. 43, 1 (1989)).

Raf genes are differentially expressed in various tissues (S.M. Storm et al., Oncogene 5, 345 (1990)). c-raf-1 has been found to be expressed ubiquitously, though absolute levels vary between tissues. A-raf-1 is present predominantly in the urogenital tissues, whereas B-Raf is most abundant in cerebrum and testis. The ubiquitous c-Raf-1 kinase is regulated by tyrosine and serine phosphorylations that result from activated growth factor receptor kinases (D.K. Morrison et al., Cell 58, 648 (1989); D.K. Morrison et al., Proc. Natl. Acad. Sci. USA 85, 8855 (1989); K.S. Kovacina et al., J. Biol. Chem. 265, 12115 (1990); P.J. Blackshear et al., J. Biol. Chem. 265, 12131 (1990); M.P. Carroll et al., J. Biol. Chem. 265, 19812 (1990); J.N. Siegel et al., J. Biol. Chem. 265, 18472 (1990); B.C. Turner et al., Proc. Natl. Acad. Sci. USA 88, 1227 (1991); M. Baccarini et al., EMBO J. 9, 3649 (1990); H. App et al., Mol. Cell. Biol. 11, 913 (1991)). Raf-1 operates downstream of Ras in mitogen signal transduction as judged by experiments using antibody microinjection (M.R. Smith et al., Nature 320, 540 (1986)), c-raf-1 antisense expression constructs (W. Kolch et al., Nature 349, 426 (1991)), dominant negative mutants (W. Kolch et al., Nature 349, 426 (1991)), and Raf revertant cells. Studies with NIH3T3 cells and brain tissue demonstrated that mitogen treatm nt

induces Raf-1 kinase activity and causes a transitory relocation of the active enzyme from the cytoplasm to the nucleus and perinuclear area (Z. Olàh et al., Exp. Brain. Res. (in press); U.R. Rapp et al., in Cold Spring Harbor Symposia on Quantitative Biology, Vol. LIII, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1988) pp. 173-184).

Raf-1 coupling has been examined in more than a dozen receptor systems and all strong mitogens stimulated Raf-1 kinase activity (U.R. Rapp, Oncogene 6, 495 (1991); D.K. Morrison et al., Cell 58, 648 (1989); D.K. Morrison et al., Proc. Natl. Acad. Sci. USA 85, 8855 (1989); K.S. Kovacina et al., J. Biol. Chem. 265, 12115 (1990); P.J. Blackshear et al., J. Biol. Chem. 265, 12131 (1990); M.P. Carroll et al., J. Biol. Chem. 265, 19812 (1990); J.N. Siegel et al., J. Biol. Chem. 265, 18472 (1990); B.C. Turner et al., Proc. Natl. Acad. Sci. USA 88, 1227 (1991); M. Baccarini et al., EMBO J. 9, 3649 (1990); H. App et al., Mol. Cell. Biol. 11, 913 (1991)), and this stimulation correlated with an increase in Raf-1 phosphorylation leading to a shift in apparent molecular weight.

SUMMARY OF THE INVENTION

It is an object of this invention to provide a method of identifying an individual at an increased risk for developing cancer.

It is another object of this invention to provide a method for determining a prognosis in patients afflicted with cancer.

It is a further object of this invention to provide a method for determining the proper course of treatment for a patient afflicted with cancer.

Further objects and advantages of the present invention will be clear from the description that follows.

In one embodiment, the present invention relates to a method of identifying an individual at an increased risk for developing cancer, comprising:

amplifying a region of the c-raf-1 gene;

analyzing products of the amplification for evidence of mutation; and

classifying an individual having one or more mutations in the region as having an increased risk for developing cancer.

In another embodiment, the present invention relates to a method for determining a prognosis in patients afflicted with cancer, comprising:

amplifying a region of the c-raf-1 gene;

analyzing products of the amplification for evidence of mutation; and

classifying patients having no mutation in said region as being less likely to suffer disease relapse or having an increased chance of survival than those patients having one or more mutations in said region.

In a further embodiment, the present invention relates to a method for determining the proper course of treatment for a patient afflicted with cancer, comprising:

amplifying a region of the c-raf-1 gene;

analyzing products of said amplification for evidence of mutation;

identifying a patient having at least one mutation in said region, which patient may require treatment proper for patients having a lesser chance of survival or decreased time to relapse; and

identifying a patient lacking mutations in said region, which patients may require treatment proper for

patients having a greater chance of survival or being less likely to suffer disease relapse.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Effect of BHT promotion on ENU tumorigenesis in NFS/n x AKR mice. The X-axis represents percent tumor induced mortality within each group, and the Y-axis reflects age in weeks. All animals were exposed to ENU transplacentally at a dose of 0.5mM/Kg of mother's body weight on day 16 of gestation (presence of vaginal plug was scored as day one). At two weeks of age mice were weaned into two separate groups and separated by sex. Both groups were housed in identical cages and supplied with food (Purina Lab Chow) and water *ad libitum*. Beginning at three weeks of age, group 2A (O) was given weekly intraperitoneal (i.p.) injection of corn oil (0.1 ml), and group 2B (◇) received weekly i.p. injections of BHT (20 mg/Kg of body weight) dissolved in corn oil. Administration of BHT reduces the mean age of mortality from approximately 20 weeks to 13, and decreases the initial age of mortality. These curves are significantly different ($p \leq 0.001$) as judged by a 2-tailed Cox test. In both groups the rate of tumorigenesis was identical for males and females.

Figure 2. Northern blot analysis of proto-oncogene expression levels in ENU induced tumors.

Figure 3. Diagnostic digestion of PCR amplified Ki-*ras* genes from ENU induced tumors. Genomic DNA was isolated from a cesium chloride gradient during RNA preparations. In each case 10 ng was amplified via PCR (95°C, 5 min. followed by 35 cycles of 95°C, 1 min. → 55°C, 1 min. → 72°, 1 min.) with 2 units of Taq I polymerase. The primers used (K1; 5'-AACTTGTGGTGGTTGGACCT-3'→ (SEQ ID NO:6) and K2; <= 3'-

GTCTTAGTGAAACACCTACT-5′ (SEQ ID NO:7)) generate a 79 b.p. product. The primer K1 ends at codon 12 and contains a mismatch from normal mouse (Ki-ras sequence its 18th nucleotide (G → C) creating a BstNI site (CCTGG) in conjunction with a normal codon 12 (GGT). Digestion of amplified product from a normal allele with BstNI produces two products of 19 and 60 b.p., whereas a mutation in one of the first two positions of codon 12 will eliminate the BstNI site. The presence of two normal alleles results in all of the product being cleaved and the presence of one mutant and one normal allele will result in only half of the product cut. In the three panels each sample was run in duplicate, uncut and cut with BstNI. F1 is DNA from an untreated NFS/n X AKR F1 mouse, and MCA5 is a murine cell line known to harbor a mutant K-ras codon 12 allele. One lymphoma (24Ly) and one cell line (117; derived from a lung adenocarcinoma) display a mutated Ki-ras codon 12 allele; however, 24Ly was a passaged tumor and examination of the original tumor showed two normal alleles indicating that this mutation was acquired during passaging.

Figure 4. c-raf-1 RNAse protection analysis of ENU induced tumors. The probe used was a 32P labeled antisense transcript from the 3′ non-coding region of a mouse c-raf-1 cDNA to the 3′ most StuI site. Hybridization of this probe with normal RNA results in a protected fragment of 1.2kb covering the region encoding the Raf-1 kinase domain. One µg of poly(A)+ RNA from each tumor and 5 µg of F1 RNA (in order to get comparable signals) was hybridized for 12 hours at 52°C with 200,000 cpm of ^{32}P labeled mouse c-raf antisense transcript. Hybrids were then digested for 30 minutes with 25 µg RNAse A and 33 units of RNAse T1 at room temperature. Digested hybrids were then incubated with 50 µg of proteinase K, phenol/chloroform extracted, ethanol precipitated, and resuspended in loading dye containing 80% formamide. Samples wer then run on 6% polyacrylamide denaturing

sequencing gels at 65 watts. Gels were vacuum dried at 80 degrees C and exposed to X-ray film. Probe is undigested probe alone; tRNA is probe hybridized to non-specific RNA; v-<u>raf</u> is probe hybridized to RNA from a v-<u>raf</u> transformed cell line and the bands detected represent single base mismatches between murine c-<u>raf</u> and v-<u>raf</u>; NFS/AKR F1 is probe hybridized with RNA from a normal (untreated) mouse; 24 LY is probe hybridized with RNA from a lymphoma; and the remaining lanes are probe hybridized with RNA isolated from lung tumors.

Figure 5. Schematic of Raf-1 protein showing sites of ENU induced mutations. CR1, CR2, and CR3 represent conserved regions 1, 2 and 3. cDNAs were made from tumor derived poly(A)+ RNA using MoMuLV reverse transcriptase. Primers (MR1 sequence and MR2 sequence) encompassing a 435 base pair region c-<u>raf</u> were then used to amplify this region via PCR. The amplification mixture was then run on 1.7% agarose gels and the 435 bp product isolated. This isolated fragment was then treated with T4 polymerase and cloned into the HincII site of M13mp18 for sequencing. Another set of primers (EMR1 sequence and EMR2 sequence) was designed containing EcoRI sites at the termini and used to amplify a 609 base pair region (encompassing the original 435 base pair region). Isolated products from these reactions were then digested with EcoRI and cloned into the EcoRI site of KS. Sequencing reactions were carried out using the Sequenase kit (USB) according to the recommended protocols for single and double stranded sequencing. Sequencing reactions were run on 6% polyacrylamide denaturing gels at 65 watts. Gels were vacuum dried at 80 degrees C and exposed to X-ray film. In each case a normal allele was also sequenced along with the mutant allele.

Figure 6. Schematic for Identifying c-raf-1 mutations. Primers 1 and 2 are shown in SEQ ID NO:8 and SEQ ID NO:9, respectively.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods that involve amplifying a region of the c-raf-1 gene (the sequence of a mouse c-raf-1 gene is shown in SEQ ID NO:10; the nucleotide and corresponding amino acid sequence of a human c-raf-1 gene is shown in SEQ ID NO:11 and SEQ ID NO:12, respectively).

In one embodiment, the present invention relates to a method of identifying an individual at an increased risk for developing cancer (preferably, lung cancer, T-cell lymphomas, renal cell carcinoma, ovarian carcinoma, and mixed parotid gland tumors) comprising: amplifying a region (preferably by using the polymerase chain reaction method(PCR) or by cloning techniques) of the c-raf-1 gene of the individual (SEQ ID NO:11)(in one preferred embodiment, the region encodes amino acids 514 to 535 of SEQ ID NO:12); analyzing products of the amplification for evidence of mutation (preferably by DNA sequencing of the region) and classifying an individual having one or more mutations in the region as having an increased risk for developing cancer. In one prefered embodiment, the region encodes amino acids 500 to 550 of SEQ ID NO:12 or amino acids 450 to 630 of SEQ ID NO:12. In another prefered embodiment, the PCR method employs a primer comprising the sequence shown in SEQ ID NO:7 and a primer comprising the sequence shown in SEQ ID NO:8. In another prefered embodiment, the method comprises the steps shown in Figure 6.

In another embodiment, the present invention relates to a method for determining a prognosis in a patient afflicted with cancer (preferably, those cancers

listed abov). The method comprises: amplifying the region of the c-raf-1 gene as described above; analyzing products of the amplification for evidence of mutation as described above; and classifying a patient having no mutation in the region as being less likely to suffer disease relapse or having an increased chance of survival than a patient having one or more mutations in the region.

In another embodiment, the present invention relates to a method for determining the proper course of treatment for a patient afflicted with cancer (preferably, those cancers listed above), comprising: amplifying a region (described above) of the c-raf-1 gene as described above; analyzing products of the amplification for evidence of mutation as described above; identifying a patient having at least one mutation in the region, which patient may require treatment proper for patients having a lesser chance of survival or decreased time to relapse; and identifying a patient lacking mutations in the region, which patients may require treatment proper for patients having a greater chance of survival or being less likely to suffer disease relapse.

Administration of therapeutic agents (cytotoxic or cytostatic) tailored to recognize the mutant Raf-1 protein but not normal Raf-1 could specifically target tumor cells for death of growth inhibition. Such agents could be comprised of cytotoxic T-cells, antibodies, and/or specifically designed chemical compounds.

The following Examples demonstrate consistent point mutations of the c-<u>raf</u>-1 proto-oncogene, within a small region of the kinase domain, in a mouse model for chemical tumor induction. This is the first demonstration of point mutated <u>raf</u> genes <u>in vivo</u>, and the first isolation of activating <u>in vivo</u> point mutations in the kinase domain of a proto-oncogene. The tumors examined

show a selective specificity for Raf-1 mutations as another family of genes, the ras proto-oncogenes which are frequently activated by point mutation in both animal and human tumors (S. Rodenhuis et al., Am. Rev. Respir. Dis. 142, S27-30; T.R. Devereux et al., Carcinogenesis 12, 299 (1991)), is not involved.

The present invention is described in further detail in the following non-limiting examples.

EXAMPLES

The following protocols and experimental details are referenced in the examples that follow:

RNA Isolation. Tumors were excised, a small portion minced in PBS (phosphate buffered saline solution) for passaging in nude mice, frozen immediately in a dry ice/ethanol bath, and stored at -70° until RNA extraction. Frozen tissues were minced on wet ice in a guanidine thiocyanate buffer (4M guanidine thiocyanate 10mM EDTA, 2% N-lauryl sarcosine, 2% beta-mercaptoethanol, 10mM Tris (pH=7.6)), disrupted in a Dounce homogenizer, and extracted three times with phenol: chloroform: isoamyl alcohol (24:24:2). Supernatants were then transferred to SW41 tubes, 100 µg of cesium chloride per ml was added to the supernatant which was then underlayed with one half saturated cesium chloride in 10mM EDTA (pH=7.0; index of refraction 1.3995-1.4000), and centrifuged at 25,000 rpm for 20 hours in a Sorvall SW-41TI rotor using a Beckman model L5-50 ultracentrifuge. Supernatants were removed and RNA pellets dissolved in 4 ml resuspension buffer (10 mM Tris-HCl pH=7.6, 5% beta-mercaptoethanol, 0.5% N-lauryl sarcosine, 10 mM EDTA), extracted once with phenol:chloroform:isoamyl alcohol, sodium acetate added to 0.12M and RNA precipatated with two volumes ethanol at -20°C overnight. Precipitates were centrifuged at 9,000 rpm in a Sorvall SS-34 rotor for 30 minutes, and pell ts

redissolved in RNA sample buffer (10 mM Tris pH=7.4, 1mM EDTA, 0.05% sodium dodecyl sulfate) and concentrations determined by absorbance at 260 nm. Poly $(A)^+$ RNA was isolated by binding to oligo dT cellulose columns in high salt (10mM Tris pH=7.4, 1mM EDTA, 0.05% SDS, 500mM NaCl), and eluting with RNA sample buffer heated to 40°C.

Northern Blotting. 5 µg poly$(A)^+$ RNA per lane was ethanol precipitated, desiccated, resuspended in loading buffer (20mM MOPS pH=6.8, 5mM sodium acetate, 1mM EDTA, 50% formamide, 6% formaldehyde), heated at 65°C for 5 min., quick chilled on wet ice for 10 min., and electrophoresed through a 0.7% agarose gel containing 2.2 M formaldehyde, 20mM MOPS [pH=6.8], 5mM sodium acetate, and 1mM EDTA. Gels were then blotted overnight onto nitrocellulose filters via capillary transfer in 20X SSC, filters were washed in 3X SSC for 10 min. and baked at 80°C for 2 hours.

Hybridizations. Filters were prehybridized at 42°C in 5X SSC, 50% formamide, 20mM sodium phosphate pH=6.8, 200 µg/ml PVP-40, 200 µg/ml ficoll 400, 200 µg/ml bovine serum albumin, and 200 µg/ml sonicated sheared salmon sperm DNA. Blots were then hybridized with 500,000 cpm/ml of random primed ^{32}P labeled probes overnight at 42°C in prehybridization solution with 5% dextran sulfate. Blots were washed with agitation in 2X SSC, 0.1% SDS at room temperature six times for 20 minutes each wash, then washed once at 45°C in 0.1X SSC for 15 minutes. Filters were exposed to X-AR 5 film at -70°C.

EXAMPLE 1
Tumor Induction

NFS female mice were mated with AKR males and pregnant females given a transplacental injection of 1-ethyl-1-nitrosourea (ENU) at a dosage of 0.5 mM/Kg

mother's body weight on day 16 of gestation, counting plug date as day one. ENU was chosen for tumor induction since it is a very potent direct acting carcinogen capable of modifying any base in vivo (Singer, B. et al., 1983. Molecular Biology of Mutagens and Carcinogens, Plenum Press, New York). ENU alkylates all tissues with roughly the same efficiency (E. Scherer et al., Cancer Lett. 46, 21 (1989)) and has a very short half life in vivo (E.M. Faustman et al., Teratology 40, 199 (1989)) allowing specific mutagenesis of tissues which are mitotically active at a particular time. NFS and AKR were chosen as parental strains based on earlier studies which showed them to be particularly susceptible to ling tumors following ENU exposure (B.A. Diwan et al., Cancer Res. 34, 764 (1974); S.L. Kauffman, JNCI 57, 821 (1976)). With this procedure nearly 100% of the offspring develop lung adenocarcinomas and approximately 70% develop, in addition, T-cell lymphomas with a mean latency of approximately 20 weeks. In order to achieve more rapid tumor development,weanling mice were treated with weekly injections of a tumor promoter, the antioxidant butylated hydroxytoluene or BHT (20mg/kg body weight dissolved in corn oil). BHT was used as it has been demonstrated to cause lung lesions and hyperplasia when injected into mice (A.A. Marino et al., Proc. Soc. Exp. Biol. Med. 140, 122 (1972); H. Witschi et al., Proc. Soc. Exp. Biol. Med., 147, 690 (1974); N. Ito et al., CRC Crit. Rev. Toxicol. 15, 109 (1984)). In the present system it nearly doubles the rate at which tumors develop. Figure 1 compares tumor induced mortality with age of animals for those receiving ENU alone, and those receiving ENU and promoted with BHT. These curves demonstrate that when BHT is given the mean age of tumor induced mortality decreases from approximately 20 weeks to around 12, and there is also a decrease in initial latency. These curves are significantly different with a confidence limit greater than 99.99% using a 2-tailed Cox test. In addition, BHT

promotion, while increasing the rate at which tumors develop, does not affect the tumor spectrum.

EXAMPLE 2

Oncogene Expression

Northern blot analysis revealed elevated levels of c-raf-1, as compared to normal tissue, in every tumor examined (Figure 2), and Western blot analysis showed that protein levels correlated with message levels (U.R. Rapp et al., in Oncogenes and Cancer, S.A. Aaronson et al., Eds. (Tokyo/VNU Scientific Press, Tokyo, 1987) pp. 55-74). In addition, in cell lines derived from primary tumors, Raf-1 protein kinase activity was shown by immune-complex kinase assays to be constitutive. Further analysis of other oncogenes revealed no consistent pattern of expression except for ras and myc family genes. In the case of the myc family, one member (either c-, N-, or L-myc) was overexpressed but never more than one. For the ras genes, at least one member (Ki-, Ha-, or N-ras), and often more than one, was expressed at high levels when compared with the normal tissue. In addition all oncogenes examined via Northern analysis exhibited full length, normal sized transcripts.

ras genes were considered likely candidates for mutational activation since oncogenic forms of Ki-ras have previously been observed in lung tumors (S. Rodenhuis et al., Am. Rev. Respir. Dis. 142, S27-30; T.R. Devereux et al., Carcinogenesis 12, 299 (1991)) and ENU is a point mutagen (Singer, B. et al., 1983. Molecular Biology of Mutagens and Carcinogens, Plenum Press, New York). A systematic analysis of various ras codons known to be involved in oncogenic activation was therefore performed. Ha-, Ki-, and N- ras were examined at codons 12, 13, and 61 for potential mutations via RNAse protection assays (R.M. Myers et al., Science 230, 1242 (1985); E. Winter et

al., Proc. Natl. Acad. Sci. USA 82, 7575 (1985)), PCR amplification followed by subsequent sequencing (F. Sanger et al., J. Mol. Biol. 13, 373 (1975)), and PCR amplification followed by diagnostic restriction digests (W. Jiang et al., Oncogene 4, 923 (1989)). PCR amplification creating diagnostic enzyme sites is a very efficient way of examining alleles for mutations at known sites and involves designing a PCR primer whose 3' end lies next to and produces a novel restriction site encompassing the codon of interest. Following amplification, PCR products from normal alleles will contain the new restriction site, while mutant alleles will not. Digestion of the product from tissue with two normal alleles results in all product being cut; however, if one allele contains a mutation, only half of the product will be digested. Figure 3 shows the results of amplification and diagnostic digestion applied to Ki-*ras* codon 12 in several tumors and cell lines. The first panel is from a set of lymphomas. F1 is DNA from a normal untreated mouse and both alleles are cut by BstNl, indicating the presence of two normal alleles. MCA5 is a murine cell line known to contain a Ki-*ras* codon 12 mutation (L.F. Parada et al., Mol. Cell. Biol. 3, 2298 (1983)), and only the amplified normal allele is cleaved. Of the five tumors shown in the second panel, one shows a mutant Ki-*ras* allele. The next panel shows some of the lung tumors tested and none of them exhibit a mutant allele, and the final panel shows tumor derived cell lines. The first three are from lymphomas and the last three from lung adenocarcinomas. One lung tumor line (#117) has a Ki-*ras* 12 mutation that was not present in the primary tumor but came up upon transplantation. This analysis has been performed with Ki, Ha and N-*ras* genes at codons 12 and 61. Of all the tumors and cell lines examined by this method for mutations of the three ras genes at codons 12 and 61, the two shown here were the only ones detected. Examination of codon 13 was done by

PCR amplification of genomic DNA surrounding codon 13 followed by cloning into KS+ (Stratagene) and double stranded sequencing. Table I summarizes the ras mutation data. The most notable point from this table is the conspicuous lack of ras mutations in these tumors. In fact the number of ras mutations is much lower than would be expected for a sampling of spontaneous tumors (S. Rodenhuis et al., Am. Rev. Respir. Dis. 142, S27-30; T.R. Devereux et al., Carcinogenesis 12, 299 (1991); J.L. Bos, Cancer Res. 49, 4682 (1989)). Having eliminated ras genes as playing a primary role in the genesis of these ENU induced tumors, c-raf-1 was investigated for possible small or point mutations.

TABLE I

Tumors and Cell Lines Positive for ras Mutations

	Codon 12		Codon 13		Codon 61	
	Tumors	Cell Lines	Tumors	Cell Lines	Tumors	Cell Lines
Ha-ras	0/10	0/6	0/6	0/2	0/10	0/6
Ki/ras	1*/10	1/6	0/6	0/2	0/6	0/2
N-ras	0/10	0/6	0/6	0/2	0/10	0/6

* This was a second passage tumor in which the original tumor did not contain a Ki-ras mutation.

Table I: Summary of mutation analysis for Ha-, Ki-, and N-ras at codons 12, 13, and 61. Each box displays the number of mutations detected, over the number of tumors and tumor derived cell lines examined via RNAse protection, sequencing or diagnostic digestion, for each of the nine codons.

EXAMPLE 3

Mutations in Raf-1

Since no point mutations had been described for raf g nes in vivo, as had been for the ras genes (E.

Santos et al., Science 223, 661 (1984); S. Rodenhuis, N. Engl. J. Med. 317, 929 (1987); M. Barbacid, Eur. J. Clin. Invest. 20, 225 (1990); F. Sanger et al., J. Mol. Biol. 13, 373 (1975)), point mutations were screened for using RNAse protection assays (R.M. Myers et al., Science 230, 1242 (1985); E. Winter et al., Proc. Natl. Acad. Sci. USA 82, 7575 (1985)). Figure 4 shows a typical protection assay using a c-raf-1 probe. In this experiment the probe used covered the 3' end of raf-1 from the 3' most StuI site to the end of the coding sequence. The first lane is a marker (pBR322 digested with HaeIII), the second shows the probe alone undigested, the third lane shows the probe hybridized to unrelated RNA in this case tRNA, the fourth lane shows hybridization with v-raf transformed cells and the lower bands represent cleavage at points where the mouse c-raf-1 gene differs from v-raf. The fifth lane shows hybridization with RNA isolated from a normal lung of an untreated F1 mouse, the next lanes are RNA isolated from several tumors. In the case of the normal RNA, only one, fully protected, band is detected while in the case of the tumors two major bands are seen after digestion. 20 out of 20 tumors analyzed in this fashion showed this extra band. These data demonstrate the following major points: 1) there is a tumor specific alteration in c-raf-1 that results in a region of non-homology recognizable by either RNAse A or T1; 2) The alterations are confined to the same region of one allele as two bands of equal size are present in the tumor lanes, and; 3) both alleles were expressed at comparable levels as both bands are of approximately equal intensity. In the assay shown 5 μg of poly(A)+ RNA was hybridized from normal tissue, and 1 μg was used from the tumors. This was necessary to get signals that could be compared on the same gel due to the overexpression of c-raf-1 in the tumors. By running these assays with various markers it was possible to estimate the approximate site of the

alteration(s) to be in the vicinity of the exon 14/exon 15 junction. In order to define the precise genetic alteration or alterations, PCR primers were designed which would generate a 600 bp fragment encompassing this region. cDNAs from tumor derived RNA were then amplified and cloned into KS+ (Stratagene) for double stranded sequencing. The sequencing results from several tumors are shown in Figure 5. The top portion of Figure 5 presents a cartoon of the mouse Raf-1 protein. There are three conserved regions CR1, CR2 and CR3 with CR3 representing the kinase domain. The probe used in the RNAse protection assays covers the indicated area, and the PCR primers amplified the bracketed region. Sequencing through this area revealed a variety of mutations just downstream of the APE site. These mutants are shown in an expanded version at the bottom of Figure 5 (See also SEQ ID NO:1 for normal mouse sequence and SEQ ID NO:2, 3, 4, and 5 for mutant sequences). These mutants were isolated from four separate tumors, and in each case a normal allele (SEQ ID NO:1) was also sequenced. Repeating the cDNA synthesis, PCR amplification, cloning and sequencing gives the same sequence and normal tissue shows no mutations demonstrating that these alterations are not artifactual. Sequence covering the amplified region has been examined and it is interesting that all of these changes occur within a very small region of the raf protein. In fact the region where these mutations occur overlaps an epitope shared by monoclonal antibodies generated against raf (W. Kolch et al., Oncogene 5, 713 (1990)), and computer modeling of the protein shows this to be a hydrophilic domain, the structure of which is predicted to be altered by these mutations. This indicates a biologically important region for the molecule and indeed the first of these mutation tested in NIH3T3 cell assays, after cloning into a retroviral expression vector (E1-neo, (G. Heidecker et al., Mol.

Cell. Biol. 10, 2503 (1990))), was found to be weakly transforming when driven by a Moloney LTR. The transformation efficiency was comparable to EC2, a previously characterized mutation of human c-raf-1 cDNA (G. Heidecker et al., Mol. Cell. Biol. 10, 2503 (1990); C. Wasylyk et al., Mol. Cell. Biol. 9, 2247 (1989)) and ~20 fold lower than the v-raf oncogene.

* * * * *

All publications mentioned hereinabove are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Rapp, Ulf R.
Storm, Stephen M.

(ii) TITLE OF INVENTION: DETECTION METHOD FOR C-RAF-1 GENES

(iii) NUMBER OF SEQUENCES: 12

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: Suite 400, 133 Carnegie Way, N.W.
(C) CITY: Atlanta
(D) STATE: Georgia
(E) COUNTRY: USA
(F) ZIP: 30303-1031

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Perryman, David G.
(B) REGISTRATION NUMBER: 33,438
(C) REFERENCE/DOCKET NUMBER: 1414.0421

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (404) 688-0770
(B) TELEFAX: (404) 688-9880

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 648 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
1               5                   10                  15
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Leu Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
            20   25   30

Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
        35   40   45

Thr Asp Ser Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
    50   55   60

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
65   70   75   80

Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
                85   90   95

Ala Val Phe Arg Leu Leu Gln Glu His Lys Gly Lys Lys Ala Arg Leu
            100   105   110

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
        115   120   125

Asp Phe Leu Asp His Val Pro Ile Thr Thr His Asn Phe Ala Arg Lys
    130   135   140

Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145   150   155   160

Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
                165   170   175

Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
            180   185   190

Leu Leu Leu Phe Pro Asn Ser Thr Val Gly Asp Ser Gly Val Pro Ala
        195   200   205

Pro Pro Ser Phe Pro Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
    210   215   220

Pro Ala Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225   230   235   240

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
                245   250   255

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu His Val
            260   265   270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
        275   280   285

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
    290   295   300

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305 310 315 320

Gly Ser Gly Thr Gln Gln Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
325 330 335

Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
340 345 350

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
355 360 365

His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
370 375 380

Glu Gln Leu Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385 390 395 400

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
405 410 415

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
420 425 430

Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
435 440 445

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
450 455 460

Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465 470 475 480

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
485 490 495

Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
500 505 510

Ala Pro Glu Val Ile Arg Met Gln Asp Asp Asn Pro Phe Ser Phe Gln
515 520 525

Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met Ala Gly
530 535 540

Glu Leu Pro Tyr Ala His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545 550 555 560

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Arg Leu Tyr Lys Asn
565 570 575

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
580 585 590

Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
595 600 605

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
610 615 620

Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625 630 635 640

Thr Ser Pro Arg Leu Pro Val Phe
645

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 648 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
1 5 10 15

Leu Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
20 25 30

Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
35 40 45

Thr Asp Ser Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
50 55 60

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
65 70 75 80

Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
85 90 95

Ala Val Phe Arg Leu Leu Gln Glu His Lys Gly Lys Lys Ala Arg Leu
100 105 110

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
115 120 125

Asp Phe Leu Asp His Val Pro Ile Thr Thr His Asn Phe Ala Arg Lys
130 135 140

Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145 150 155 160

Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
165 170 175

Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
180 185 190

Leu Leu Leu Phe Pro Asn Ser Thr Val Gly Asp Ser Gly Val Pro Ala
195 200 205

Pro Pro Ser Phe Pro Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
210 215 220

Pro Ala Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225 230 235 240

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
245 250 255

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu His Val
260 265 270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
275 280 285

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
290 295 300

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305 310 315 320

Gly Ser Gly Thr Gln Gln Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
325 330 335

Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
340 345 350

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
355 360 365

His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
370 375 380

Glu Gln Leu Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385 390 395 400

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
405 410 415

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
420 425 430

Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
435 440 445

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
450 455 460

Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465 470 475 480

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
485 490 495

Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
500 505 510

Ala Pro Glu Val Val Arg Met Gln Asp Asp Asn Pro Phe Ser Phe Gln
515 520 525

Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met Ala Gly
530 535 540

Glu Leu Pro Tyr Ala His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545 550 555 560

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Arg Leu Tyr Lys Asn
565 570 575

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
580 585 590

Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
595 600 605

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
610 615 620

Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625 630 635 640

Thr Ser Pro Arg Leu Pro Val Phe
645

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 648 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
1 5 10 15

Leu Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
20 25 30

Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
35 40 45

```
Thr Asp Ser Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
    50                  55                  60

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
65                  70                  75                  80

Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
                85                  90                  95

Ala Val Phe Arg Leu Leu Gln Glu His Lys Gly Lys Lys Ala Arg Leu
            100                 105                 110

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
        115                 120                 125

Asp Phe Leu Asp His Val Pro Ile Thr Thr His Asn Phe Ala Arg Lys
    130                 135                 140

Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145                 150                 155                 160

Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
                165                 170                 175

Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
            180                 185                 190

Leu Leu Leu Phe Pro Asn Ser Thr Val Gly Asp Ser Gly Val Pro Ala
        195                 200                 205

Pro Pro Ser Phe Pro Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
    210                 215                 220

Pro Ala Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225                 230                 235                 240

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
                245                 250                 255

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu His Val
            260                 265                 270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
        275                 280                 285

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
    290                 295                 300

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305                 310                 315                 320

Gly Ser Gly Thr Gln Gln Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
                325                 330                 335
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Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
            340                 345                 350

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
        355                 360                 365

His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
    370                 375                 380

Glu Gln Leu Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385                 390                 395                     400

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
                405                 410                     415

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
            420                 425                 430

Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
        435                 440                 445

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
    450                 455                 460

Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465                 470                 475                     480

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
                485                 490                     495

Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
            500                 505                 510

Ala Pro Glu Val Ile Arg Met Gln Asp Asn Asn Pro Phe Ser Phe Gln
        515                 520                 525

Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met Ala Gly
    530                 535                 540

Glu Leu Pro Tyr Ala His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545                 550                 555                     560

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Arg Leu Tyr Lys Asn
                565                 570                     575

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
            580                 585                 590

Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
        595                 600                 605

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
    610                 615                 620
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Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625 630 635 640

Thr Ser Pro Arg Leu Pro Val Phe
645

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 648 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
1 5 10 15

Leu Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
20 25 30

Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
35 40 45

Thr Asp Ser Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
50 55 60

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
65 70 75 80

Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
85 90 95

Ala Val Phe Arg Leu Leu Gln Glu His Lys Gly Lys Lys Ala Arg Leu
100 105 110

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
115 120 125

Asp Phe Leu Asp His Val Pro Ile Thr Thr His Asn Phe Ala Arg Lys
130 135 140

Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145 150 155 160

Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
165 170 175

Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
180 185 190

Leu Leu Leu Phe Pro Asn Ser Thr Val Gly Asp Ser Gly Val Pro Ala
195 200 205

```
Pro Pro Ser Phe Pro Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
    210                 215                 220

Pro Ala Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225                 230                 235                 240

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
                245                 250                 255

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu His Val
            260                 265                 270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
        275                 280                 285

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
    290                 295                 300

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305                 310                 315                 320

Gly Ser Gly Thr Gln Gln Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
                325                 330                 335

Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
            340                 345                 350

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
        355                 360                 365

His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
    370                 375                 380

Glu Gln Leu Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385                 390                 395                 400

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
                405                 410                 415

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
            420                 425                 430

Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
        435                 440                 445

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
    450                 455                 460

Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465                 470                 475                 480

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
                485                 490                 495
```

Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
500 505 510

Ala Pro Glu Val Ile Arg Met Gln Asp Asp Asn Pro Phe Ser Ser Gln
515 520 525

Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met Ala Gly
530 535 540

Glu Leu Pro Tyr Ala His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545 550 555 560

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Arg Leu Tyr Lys Asn
565 570 575

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
580 585 590

Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
595 600 605

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
610 615 620

Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625 630 635 640

Thr Ser Pro Arg Leu Pro Val Phe
645

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 648 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
1 5 10 15

Leu Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
20 25 30

Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
35 40 45

Thr Asp Ser Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
50 55 60

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
65 70 75 80

```
Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
                85                  90                  95

Ala Val Phe Arg Leu Leu Gln Glu His Lys Gly Lys Lys Ala Arg Leu
            100                 105                 110

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
        115                 120                 125

Asp Phe Leu Asp His Val Pro Ile Thr Thr His Asn Phe Ala Arg Lys
    130                 135                 140

Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145                 150                 155                 160

Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
                165                 170                 175

Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
            180                 185                 190

Leu Leu Leu Phe Pro Asn Ser Thr Val Gly Asp Ser Gly Val Pro Ala
        195                 200                 205

Pro Pro Ser Phe Pro Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
    210                 215                 220

Pro Ala Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225                 230                 235                 240

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
                245                 250                 255

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu His Val
            260                 265                 270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
        275                 280                 285

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
    290                 295                 300

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305                 310                 315                 320

Gly Ser Gly Thr Gln Gln Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
                325                 330                 335

Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
            340                 345                 350

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
        355                 360                 365
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His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
370 375 380

Glu Gln Leu Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385 390 395 400

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
405 410 415

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
420 425 430

Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
435 440 445

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
450 455 460

Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465 470 475 480

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
485 490 495

Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
500 505 510

Ala Pro Glu Val Ile Arg Met Gln Asp Asp Asn Pro Phe Ser Phe Gln
515 520 525

Ser Thr Cys Thr Phe Tyr Gly Ile Val Leu Tyr Glu Leu Met Ala Gly
530 535 540

Glu Leu Pro Tyr Ala His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545 550 555 560

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Arg Leu Tyr Lys Asn
565 570 575

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
580 585 590

Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
595 600 605

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
610 615 620

Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625 630 635 640

Thr Ser Pro Arg Leu Pro Val Phe
645

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AACTTGTGGT GGTTGGACCT 20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCATCCACAA AGTGATTCTG 20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGGAGACCAA GTTTCAGATG 20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1947 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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CGGGCCTCAG ATGATGGCAA GCTCACGGAT TCTTCTAAGA CAAGCAATAC TATCCGGGTT  180
TTCTTGCCGA ATAAGCAAAG GACTGTGGTC AATGTGCGGA ATGGAATGAG CTTACATGAC  240
TGCCTTATGA AAGCTCTGAA GGTGAGAGGC CTGCAGCCAG AGTGCTGTGC AGTGTTCAGA  300
CTTCTCCAGG AACACAAAGG TAAGAAAGCA CGCTTAGATT GGAACACCGA TGCCGCCTCT  360
CTGATTGGAG AAGAACTGCA AGTGGATTTT TTGGATCATG TTCCCATCAC AACTCACAAC  420
TTTGCTCGGA AAACGTTCCT GAAGCTTGCA TTCTGTGACA TCTGTCAGAA GTTCCTGCTA  480
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CCTACTATGT GTGTGGACTG GAGTAATATC AGACAGCTCT TGCTGTTTCC AAATTCCACT  600
GTTGGTGACA GTGGAGTCCC AGCACCACCT TCTTTCCCAA TGCGTCGGAT GCGAGAATCT  660
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AACACCTCCA GCCCTTCCTC AGAAGGTTCC CTCTCCCAGA GGCAGAGGTC AACGTCCACT  780
CCCAATGTCC ACATGGTCAG CACCACCCTG CATGTGGACA GCAGGATGAT TGAGGATGCA  840
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GGTCCAACAG GCTGGTCACA GCCCAAAACC CCCGTGCCAG CACAAAGAGA GCGGGCACCA  960
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CAGATCCTGT CTTCCATCGA GCTGCTTCAG CACTCTCTGC CGAAAATCAA CAGGAGCGCC 1860

TCTGAGCCTT CCCTGCATCG GGCAGCTCAC ACTGAGGACA TCAATGCTTG CACGCTGACT 1920

ACATCCCCAA GGCTACCAGT CTTCTAG . 1947

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1944

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
85 90 95

GCA GTG TTC AGA CTT CTC CAC GAA CAC AAA GGT AAA AAA GCA CGC TTA 336
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Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
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Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145 150 155 160

AAT GGA TTT CGA TGT CAG ACT TGT GGC TAC AAA TTT CAT GAG CAC TGT 528
Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
165 170 175

AGC ACC AAA GTA CCT ACT ATG TGT GTG GAC TGG AGT AAC ATC AGA CAA 576
Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
180 185 190

CTC TTA TTG TTT CCA AAT TCC ACT ATT GGT GAT AGT GGA GTC CCA GCA 624
Leu Leu Leu Phe Pro Asn Ser Thr Ile Gly Asp Ser Gly Val Pro Ala
195 200 205

CTA CCT TCT TTG ACT ATG CGT CGT ATG CGA GAG TCT GTT TCC AGG ATG 672
Leu Pro Ser Leu Thr Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
210 215 220

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Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu Pro Val
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Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
275 280 285

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GTA TCT GGG ACC CAG GAG AAA AAC AAA ATT AGG CCT CGT GGA CAG AGA 1008

Val Ser Gly Thr Gln Glu Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
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GAT TCA AGC TAT TAT TGG GAA ATA GAA GCC AGT GAA GTG ATG CTG TCC 1056

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CAC GGA GAT GTT GCA GTA AAG ATC CTA AAG GTT GTC GAC CCA ACC CCA 1152

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CTG CAT GTC CAG GAG ACC AAG TTT CAG ATG TTC CAG CTA ATT GAC ATT 1344

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ATC CAT AGA GAC ATG AAA TCC AAC AAT ATA TTT CTC CAT GAA GGC TTA 1440

Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
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AGT GGT TCT CAG CAG GTT GAA CAA CCT ACT GGC TCT GTC CTC TGG ATG 1536

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(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 648 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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Thr Asp Pro Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
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Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
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Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
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Ala Val Phe Arg Leu Leu His Glu His Lys Gly Lys Lys Ala Arg Leu
            100                 105                 110

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
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Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
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Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
                165                 170                 175
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Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
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Leu Leu Leu Phe Pro Asn Ser Thr Ile Gly Asp Ser Gly Val Pro Ala
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Pro Val Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
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Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
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Val Ser Gly Thr Gln Glu Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
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610 615 620

Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625 630 635 640

Thr Ser Pro Arg Leu Pro Val Phe
645

WHAT IS CLAIMED IS:

1. A method of identifying an individual at an increased risk for developing cancer, comprising:

amplifying a region of the c-raf-1 gene of said individual;

analyzing products of said amplification for evidence of mutation; and

classifying an individual having one or more mutations in said region as having an increased risk for developing cancer.

2. The method according to claim 1, wherein said region encodes at least amino acids 514 to 535 of SEQ ID NO:12.

3. The method according to claim 2, wherein said region encodes at least amino acids 500 to 550 of SEQ ID NO:12.

4. The method according to claim 3, wherein said region encodes at least amino acids 450 to 630 of SEQ ID NO:12.

5. The method according to claim 1, wherein said products are analyzed by DNA sequencing.

6. The method according to claim 1, wherein said amplification is effected using a polymerase chain reaction (PCR).

7. The method according to claim 6, wherein said PCR employs a primer comprising SEQ ID NO:7 and a primer comprising SEQ ID NO:8.

8. A method for determining a prognosis in patients afflicted with cancer, comprising:

amplifying a region of the c-raf-1 gene of said individual;

analyzing products of said amplification for evidence of mutation; and

classifying a patient having no mutation in said region as being less likely to suffer disease relapse or having an increased chance of survival than a patient having one or more mutations in said region.

9. The method according to claim 8, wherein said region encodes at least amino acids 514 to 535 of SEQ ID NO:12.

10. The method according to claim 9, wherein said region encodes at least amino acids 500 to 550 of SEQ ID NO:12.

11. The method according to claim 10, wherein said region encodes at least amino acids 450 to 630 of SEQ ID NO:12.

12. The method according to claim 9, wherein said products are analyzed by DNA sequencing.

13. The method according to claim 9, wherein said amplification is effected using polymerase chain reaction (PCR).

14. The method according to claim 13, wherein said PCR employs a primer comprising SEQ ID NO:7 and a primer comprising SEQ ID NO:8.

15. A method for determining the proper course of treatment for a patient afflicted with cancer, comprising:

amplifying a region of the c-raf-1 gene of said patient;

analyzing products of said amplification for evidence of mutation;

identifying a patient having at least one mutation in said region, which patient may require treatment proper for a patient having a lesser chance of survival or decreased time to relapse; and

identifying a patient lacking mutations in said region, which patient may require treatment proper for a patient having a greater chance of survival or being less likely to suffer disease relapse.

16. The method according to claim 15, wherein said region encodes at least amino acids 514 to 535 of SEQ ID NO:12.

17. The method according to claim 16, wherein said region encodes at least amino acids 500 to 550 of SEQ ID NO:12.

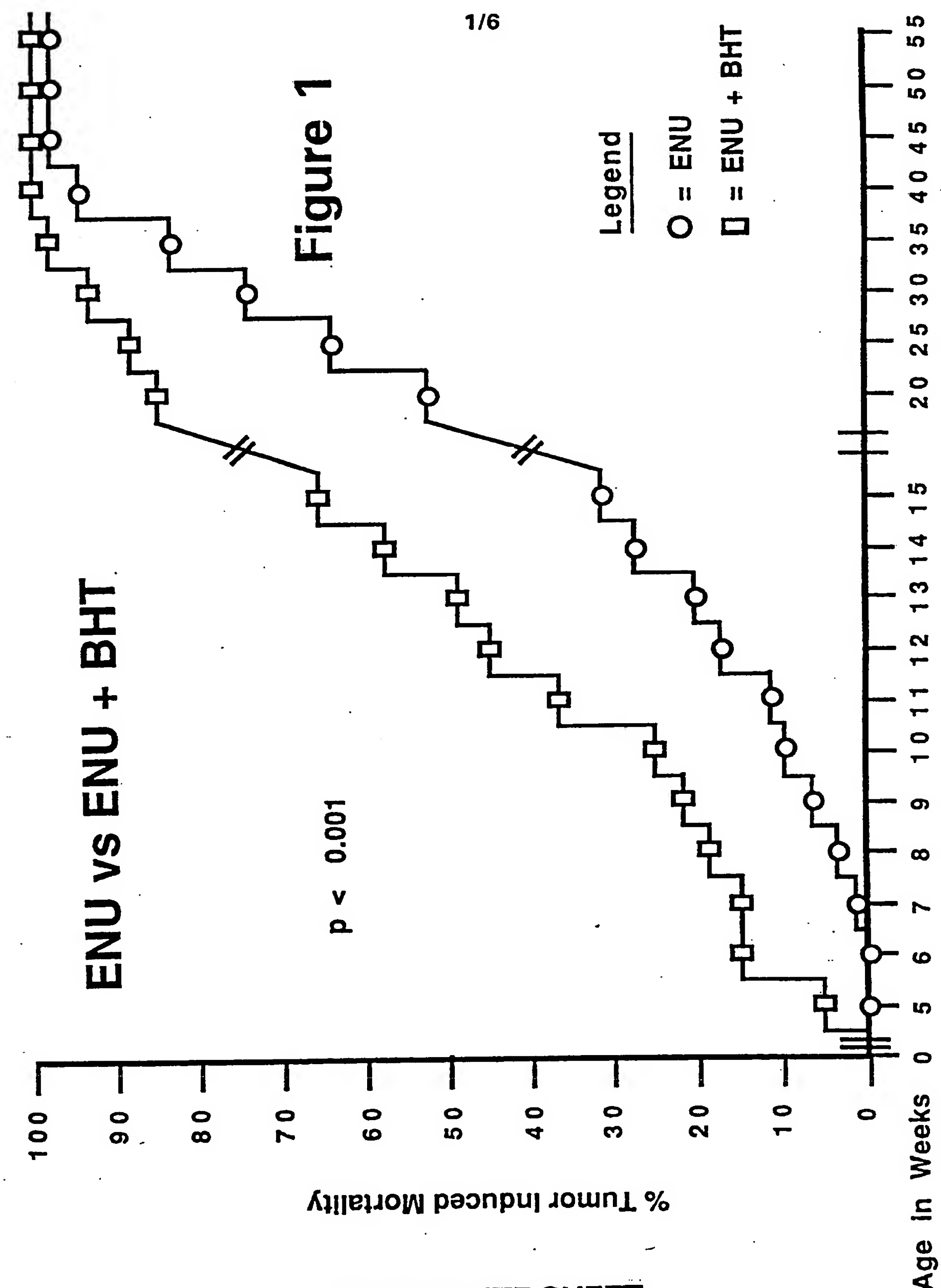
18. The method according to claim 17, wherein said region encodes at least amino acids 450 to 630 of SEQ ID NO:12.

19. The method according to claim 16, wherein said products are analyzed by DNA sequencing.

20. The method according to claim 16, wherein said amplification is effected using a polymerase chain reaction (PCR).

21. The method according to claim 20, wherein said PCR employs a primer comprising SEQ ID NO:7 and a primer comprising SEQ ID NO:8.

1/6


ENU vs ENU + BHT
p < 0.001
Legend
O = ENU
□ = ENU + BHT
% Tumor Induced Mortality
100
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80
70
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50
40
30
20
10
0
Age In Weeks
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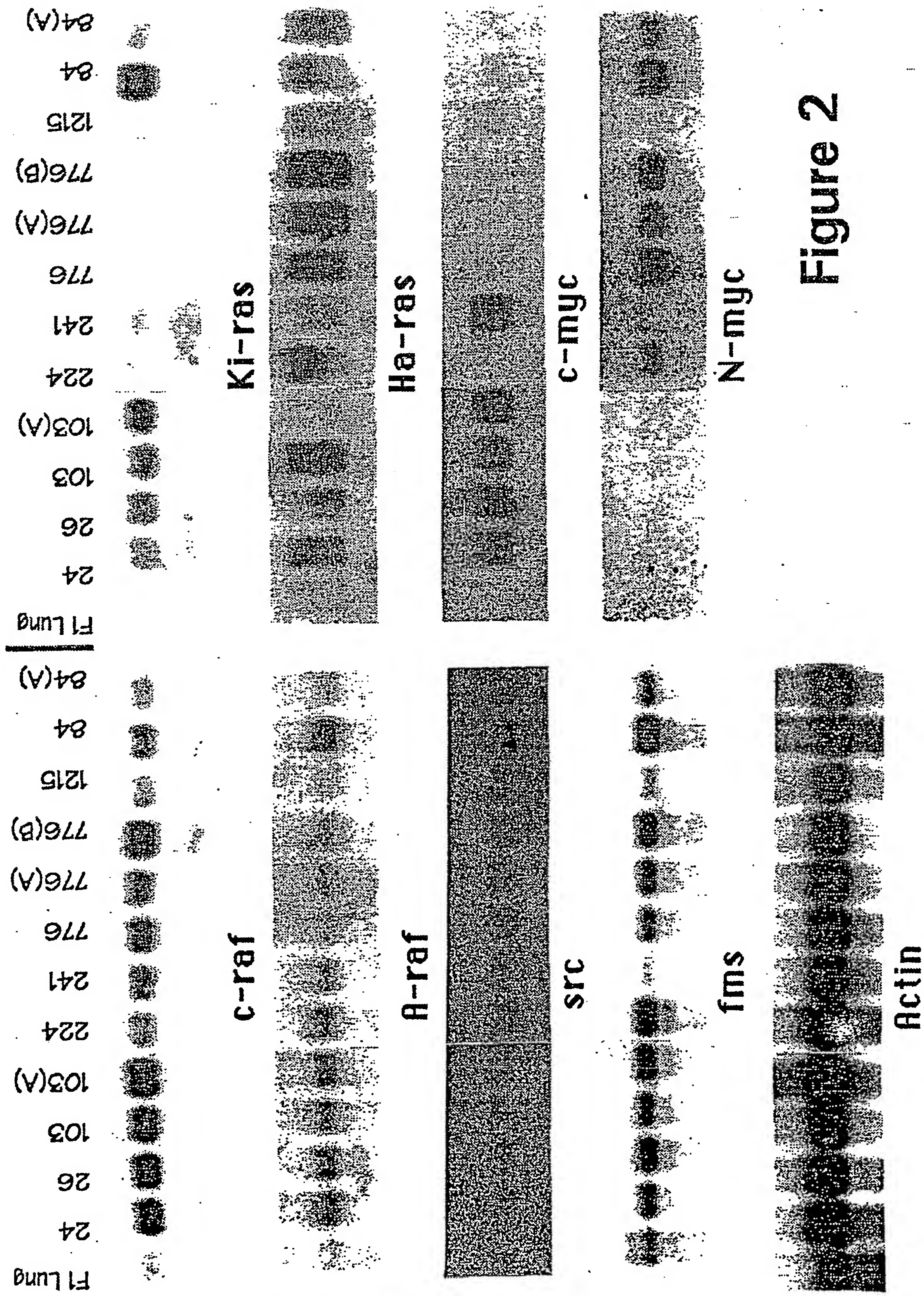
Figure 1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26
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103(A)
224
241
776
776(A)
776(B)
1215
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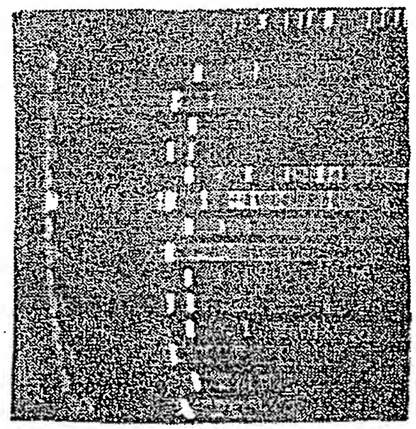
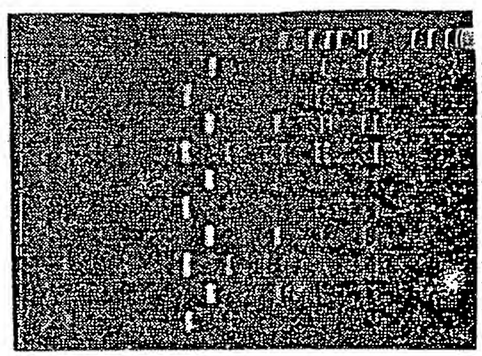
c-myc
N-myc
c-raf
A-raf
src
fms
Actin

Figure 2

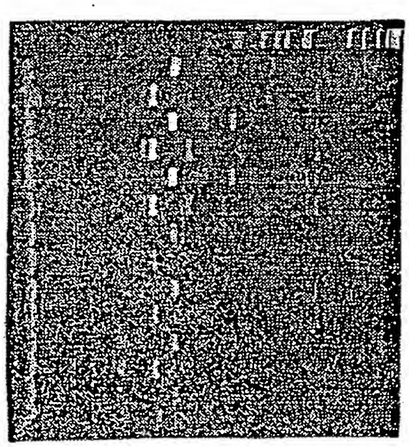
Figure 3



Lymphomas

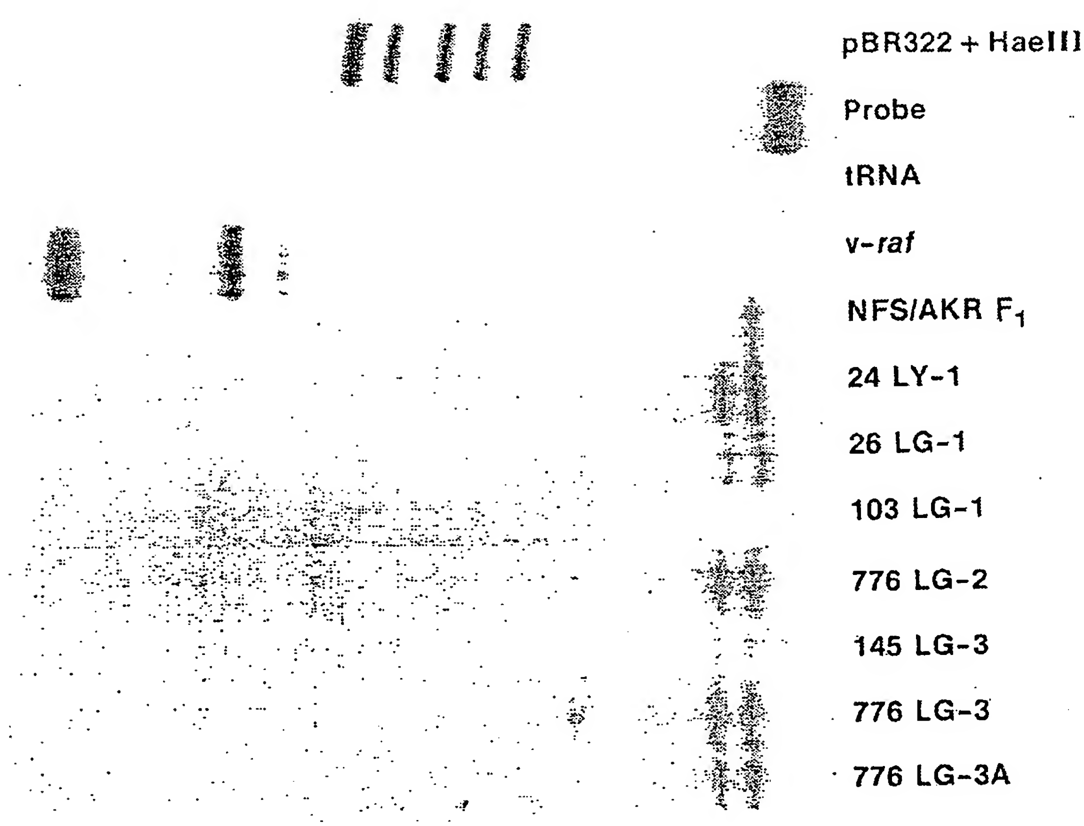


Lung Tumors



Cell Lines

Figure 4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Probe
tRNA
v-*raf*
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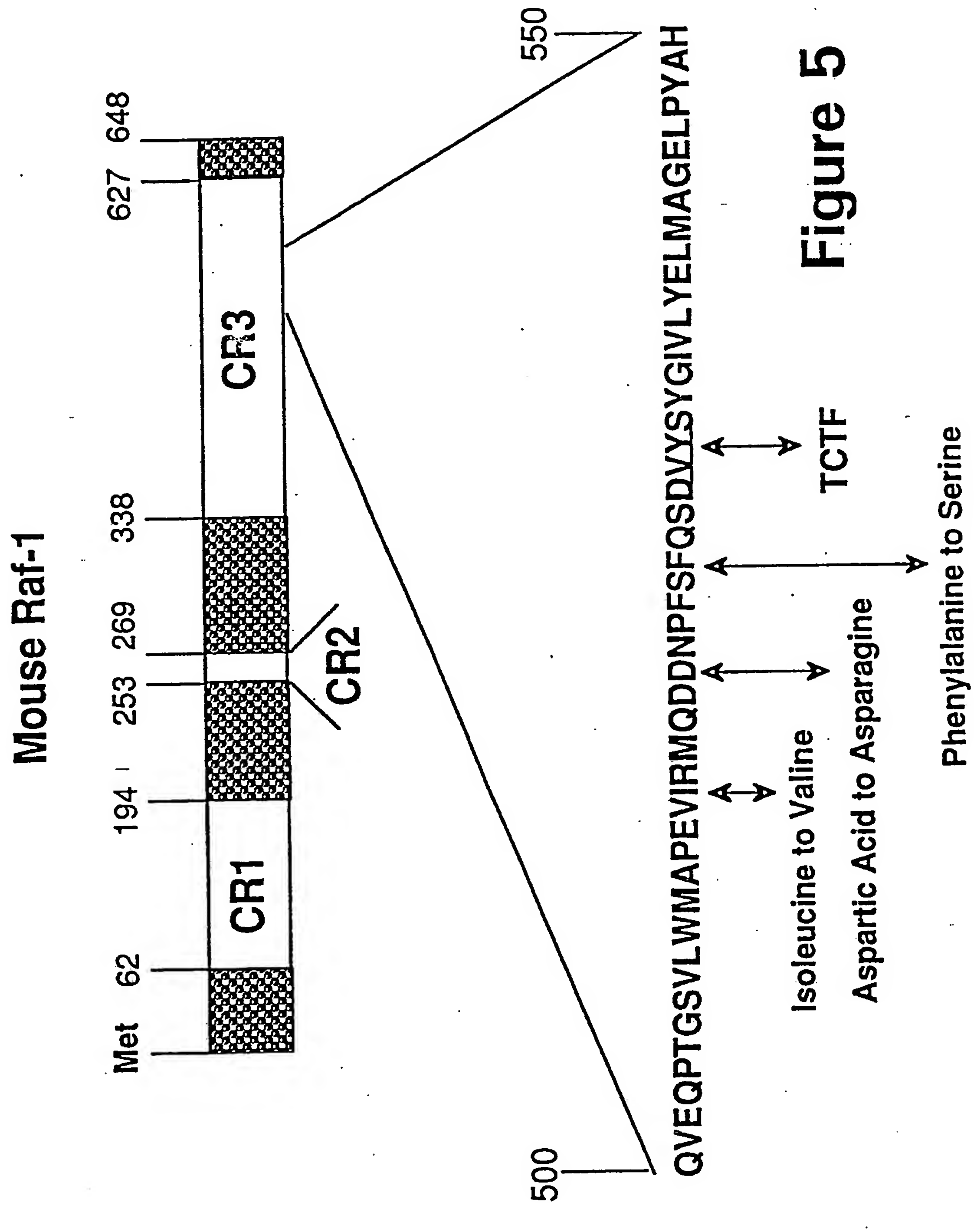
776 LG-3A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Met
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269
338
627
648
CR1
CR2
CR3
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550
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TCTF
Isoleucine to Valine
Aspartic Acid to Asparagine
Phenylalanine to Serine

Figure 5

Figure 6

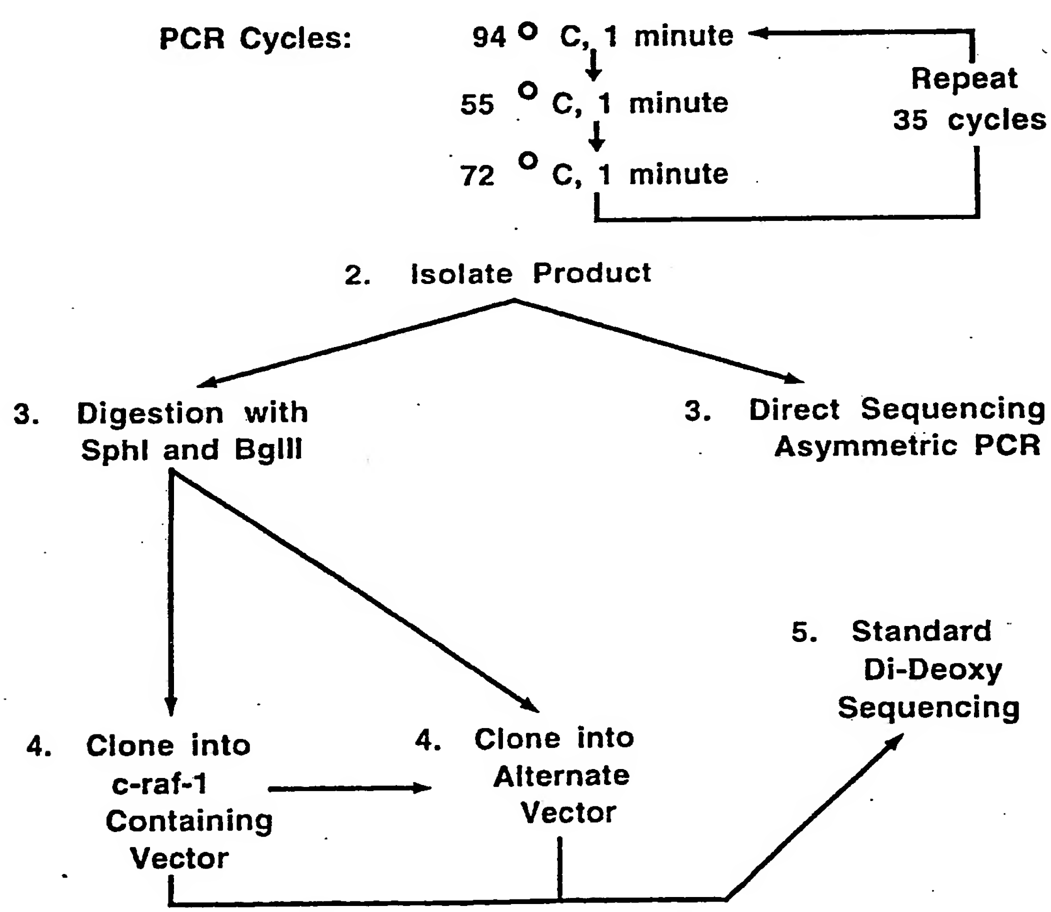
1. Polymerase Chain Reaction (PCR) Amplification of Target DNA Either Genomic DNA or cDNA

Nucleotide 1307 of coding — Nucleotide 1326 of coding

Primer 1 = 5'- AGGAGACCAAGTTTCAGATG -3'

Nucleotide 1915 of coding — Nucleotide 1896 of coding

Primer 2 = 5'- GCGTGCAAGCATTGATATCC -3'



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 92/07817

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	WO, A1, 9112343 (CETUS CORPORATION) 22 August 1991, see the whole document --	1-21
A	WO, A1, 9105064 (USA, REPR. BY THE SECR.,D. OF HEALTH/HUMAN S.) 18 April 1991, see the whole document --	1-21
A	WO, A1, 9009456 (VIKTOR BALAZS) 23 August 1990, see the whole document --	1-21
A	Chemical Abstracts, volume 104, no. 19, 12 May 1986, (Columbus, Ohio, US), Bonner Tom I. et al.: "The complete coding sequence of the human raf oncogene and the corresponding structure of the c-raf-1 gene", see page 141, abstract 162785c, & Nucleic Acids Res. 1986, 14(2), 1009-1015	1-21

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14th December 1992	12.01.93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Mikael G:son Bergstrand

International application No.
PCT/US92/07817

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Claims 1-21
Diagnostic methods, c.f. PCT Rule 39(iv). Nevertheless, a search has been made concerning the subject matter of the application (the analytical method).

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 92/07817

SA 64903

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 02/12/92
The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9112343	22/08/91	AU-D-	7758991	03/09/91
		EP-A-	0514501	25/11/92
WO-A1- 9105064	18/04/91	AU-D-	6606190	28/04/91
		CA-A-	2067114	03/04/91
		EP-A-	0494968	22/07/92
WO-A1- 9009456	23/08/90	EP-A-	0458831	04/12/91

For more details about this annex : see fficial Journal of the European patent Office, No. 12/82

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